Toxicology in Vitro 27 (2013) 580–587

Contents lists available at SciVerse ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

BDE-99 congener induces cell death by apoptosis of human hepatoblastoma cell line – HepG2

A.O. Souza [a,*], L.C. Pereira [b], D.P. Oliveira [b], D.J. Dorta [a]

[a] Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Departamento de Química, Universidade de São Paulo, Av. Bandeirantes, 3900, CEP:14040-901, Bairro Monte Alegre, Ribeirão Preto, São Paulo, Brazil

[b] Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Departamento de Análises Clínicas, Toxicológicas e Bromatológicas, Universidade de São Paulo, Av. Bandeirantes, 3900, CEP:14040-903, Bairro Monte Alegre, Ribeirão Preto, São Paulo, Brazil

ARTICLE INFO

Article history:
Received 7 May 2012
Accepted 21 September 2012
Available online 2 November 2012

Keywords:
BDE-99
HepG2 cells
Apoptosis
Cell death
ROS

ABSTRACT

Polybrominated Diphenyl Ethers (PBDEs) are an important class of flame retardants with a wide range of toxic effects on biotic and abiotic systems. The toxic mechanisms of PBDEs are still not completely understood because there are several different congeners with different chemical and biological characteristics. BDE-99 is one of these, widely found in the environment and biological samples, showing evidence of neurotoxic and endocrine disruption activities, but with little information about its action mechanism described in the current literature. This work investigated the effects of BDE-99 on the HepG2 cell line in order to clarify its toxic mechanism, using concentrations of 0.5–25 μM (24 and 48 h). Our results showed that BDE-99 could cause cell death in the higher concentrations, its activity being related to a decrease in mitochondrial membrane potential and an accumulation of ROS. It was also shown that BDE-99 induced the exposure of phosphatidylserine, caspases 3 and 9 activation and DNA fragmentation in HepG2 cells, without causing the release of LDH. Thus it was shown that BDE-99 could cause HepG2 cell death by apoptosis, suggesting its toxicity to the human liver.

1. Introduction

Scientific and technological development brings benefits and advantages to our modern lifestyle. Innovation is currently a necessity due to the great demand for new consumer products, but this also brings serious consequences to the current and future generations due to factors such as air, soil and water pollution as related to the release of several chemicals potentially harmful to the environment and human health.

Amongst these compounds are the brominated flame retardants (BFRs) that represent a class of contaminants widely used in consumer products due to their high efficiency in inhibiting or minimizing the effects caused by fires, and their low cost; representing 25% of the world market of flame retardants (Hardy, 1999). However it has been shown that they persist in the environment and show high bioaccumulation potential, being classified as persistent organic pollutants (POPs).

Polybrominated Diphenyl Ethers (PBDEs) are a class of BFRs used as additives in plastics, textiles, electronic circuits and equipments, building materials and many other consumer goods. They are added during the manufacture of various products in daily use, but no effective chemical bonds occurred during the process which would cause their release into the environment during manipulation or improper disposal (Mcdonald, 2002).

The bioaccumulation potential of PBDEs and their persistence in the environment are due to their lipophilicity, and high levels of these compounds have been detected in samples of animal fats, blood, placenta and breast milk. (Covaci et al., 2009; Hites, 2004; Li et al., 2008; Ma et al., 2012; Shen et al., 2010; Letcher et al., 2010; Toms et al., 2007). The main contamination routes for humans are house dust and contaminated foods (Branchi et al., 2003; Talsness, 2008).

Amongst the effects described as caused by exposure to PBDEs, there is evidence of a neurotoxic potential (Branchi et al., 2003; Madia et al., 2004; Verner et al., 2011) and changes in the endocrine system, by acting on hormone receptors such as estrogen and progesterone, and decreasing the levels of the thyroid hormones (Costa and Giordano, 2007; Costa et al., 2008; Madia et al., 2004; McDonald, 2002; Zhang et al., 2008). They have also being related to the development of liver toxicity and thyroid cancer (Albina et al., 2010; Hu et al., 2007; Zhang et al., 2008), but the mechanisms underlying these effects are still not completely understood.

2,2′,4,4′ Tetrabromodiphenyl ether (BDE-47) and 2,2′,4,4′,5 pentabromodiphenyl ether (BDE-99) are the most commonly found congeners in environmental samples and biological systems, and show high levels of toxicity. *In vitro* investigations have shown

* Corresponding author. Tel.: +55 16 36020472; fax: +55 16 36024838.
E-mail address: lekasouza@gmail.com (A.O. Souza).

0887-2333
http://dx.doi.org/10.1016/j.tiv.2012.09.022

that some PBDE congeners, such as BDE-47 and BDE-209, present cytotoxic potential in several cell lines such as HepG2 (Madia et al., 2004; Jing et al., 2010; Weihong et al., 2008; Hu et al., 2007, 2009; Yan et al., 2011), however little is known about the initial events that trigger these effects. In addition, there are no data about the effects of BDE-99 on HepG2 cells, a fact that makes it difficult to compare the different congeners. Therefore an investigation of the toxic effects of congeners with different amounts of bromine substituents is required, in order to better understand the mechanism of action of this class of compounds.

Reports have demonstrated that BDE-99 is found mainly in the liver of humans or animals, and is related to the development of hepatoxicity (Albina et al., 2010) which can be due to the original compound or to the metabolites that can be more toxic than the original congener (Gandhi et al., 2011). So, hepatic cell models are important experimental tools to investigate their action mechanism.

HepG2 cells are derived from human hepatoblastoma and are widely used in several *in vitro* assays (Knasmuller et al., 1998). Due to the need for more data about the toxicity of the PBDEs and particularly about the consequences of exposure to BDE-99, this work proposed to investigate its effects on HepG2 cells.

2. Materials and methods

2.1. Cell culture

HepG2 cells (American Type Culture Collection, n° HB8065) were cultured in "Minimum Essential Medium" MEM supplemented with 10% fetal calf serum in an atmosphere containing 5% CO_2 at 37 °C until the cells reached a confluence suitable for starting testing. After this procedure, adequate amounts of cells were plated and incubated for 24 h to ensure good adhesion before initiating the experiments.

2.2. Reagents

Congener BDE-99 was purchased from AccuStandard (New Haven, USA). Sulforhodamine B (SRB), 3 (4,5 dimethylthiazol-2-il)-2,5 Diphenyltetrazolium Bromide (MTT), Dimethyl Sulfoxide (DMSO), Propidium Iodide (PI), tert-Butyl hydroperoxide solution (TBHP), Triton X-100 and bisBenzimide H 33342 trihydrochloride (Hoechst 33342) were purchased from Sigma–Aldrich (EUA). Tetramethylrhodamine Methyl Ester (TMRM), Fetal Bovine Serum (GIBCO), 5,6-Chloromethyl-2′,7′-Dichlorodihydrofluorescein Diacetate, Acetyl Ester (CM-H_2DCFDA) and "Minimum Essential Medium" MEM (GIBCO) were purchased from Invitrogen (USA). Annexin V-FITC was purchased from Proteimax (Brazil) and the Cisplatin Solution (Citoplax®) from Bergamo (Brazil). All other reagents were of the highest commercial degree. The amounts of Dimethyl Sulfoxide (DMSO) required to dissolve the BDE-99 had no effect on the assays. All stock solutions were prepared using glass-distilled deionized water.

2.3. Cell proliferation assay

In order to evaluate the effects of several concentrations of the BDE-99, cell proliferation was assessed using the SRB colorimetric assay according to Skehan et al. (1990).

Briefly, HepG2 cells were cultured to a density of 5×10^4 cells. The cultures were then exposed to BDE-99 at final concentrations ranging from 0.5 to 25 μM. Each sample had at least three replicates and was cultured for 24 and 48 h. The media were then discarded and the cells washed once with phosphate buffer saline (PBS) and twice with distilled water to remove the salts.

After this procedure, the cells were dried at room temperature and subsequently fixed in a 1% methanol in 1% acetic acid solution for 2 h. The fixed cells were stained with a 0.5% SRB in 1% acetic acid solution, and then washed with a 1% acetic acid solution to remove the excess probe. The SRB attached to the cell membranes was extracted using 1 ml of a 10 mM Tris solution, pH 10.0. The absorbance of the dye was then measured at a wavelength of 540 nm in a microplate reader (Varian Cary 50MPR, Varian, USA).

2.4. Cell viability assay (MTT assay)

Cell viability was assessed using a (4,5 dimethylthiazole-2-il)-2,5 diphenyltetrazolium bromide dye, according to Denizot and Lang (1986).

HepG2 cells were seeded with a density of 1×10^5 cells and exposed to BDE-99 at final concentrations ranging from 0.5 to 25 μM. At least three replicates were made for each sample and cultured for 24 and 48 h. The cells were subsequently incubated with a 0.5% MTT (5 mg/mL) solution in an atmosphere containing 5% CO_2 at 37 °C for 3 h. After this period, the medium in the wells was discarded and the formazan crystals formed dissolved in a DMSO solution in 0.2 M glycine buffer, pH 10.2. The final absorbance was evaluated at 570 nm wavelength in a microplate reader (Varian Cary 50MPR, Varian, USA). The results were shown as the percentage difference from the control group.

2.5. Mitochondrial membrane potential

Indications of cell damage can be evaluated by mitochondrial depolarization, since the collapse of the membrane potential compromises the cell energy and consequently damages cell integrity. Mitochondrial depolarization can be measured using the fluorescent dye TMRM, a cation compound permeable to cell membranes, which is rapidly sequestered by the mitochondria of intact cells, and produces a stoichiometric relationship between the fluorescence and the mitochondrial membrane potential (Imberti et al., 1993).

The HepG2 cells were cultured to a density of 1×10^5 cells and then exposed to BDE-99 at final concentrations ranging from 0.5 to 25 μM. Each sample was tested with at least three replicates. The cells were then washed with PBS, trypsinised and incubated with a 6.6 μM TMRM solution at 37 °C for 30 min. The samples were subsequently lysed with a 0.1% Triton X-100 solution (v/v) and the TMRM captured and retained by the mitochondria measured at the excitation and emission wavelengths of 485 and 590 nm, respectively, using a F-4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). The results are shown as the percentage of fluorescence in relation to the control group.

2.6. Accumulation of reactive oxygen species (ROS)

The accumulation of ROS can be evaluated using CM-H_2DCFDA, a reactive oxygen species indicator that becomes fluorescent in the presence of intracellular oxidation (Chernyak et al., 2006).

The HepG2 cells were cultured to a density of 1×10^5 cells. After incubation with BDE-99, the cells were further incubated with a 2 mM CM-H_2DCF-DA solution at 37 °C for 1 h. The fluorescence of the CM-H_2DCFDA was subsequently measured using a F-4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) the excitation and emission wavelengths of at 503 and 528 nm, respectively. The results were shown as the difference from the control group. A tert-butyl hydroperoxide (100 μM) solution was used to induce oxidative stress.

2.7. The exposure of phosphatidylserine on the outer cell membrane (Annexin V assay)

The exposure of phosphatidylserine on the outer cell membrane is the first sign that indicates the cells are undergoing apoptosis. Annexin-V is a protein with a high affinity for membrane phospholipids, and its use combined with a fluorescent agent has been widely used to assess phosphatidylserine externalization during the apoptotic process (Zhivotovsky et al., 1999).

The HepG2 cells were cultured to a density of 1×10^5 cells and then treated with BDE-99 at the same concentrations that showed greater effects in the viability and proliferation cell assays. Each sample was tested with at least three replicates. The cells were then incubated with a 0.25 μg/ml FITC-Annexin-V solution and a 0.5 μg/ml Propidium Iodide solution and incubated for 15 min. The cells were analyzed using a BD-FACSCANTO™ flow cytometer (BD Bioscience, CA, USA) and BD-FACSDIVA software (BD Bioscience, CA, USA).

2.8. Cell membrane integrity

The cell membrane integrity was assessed by measuring the lactate dehydrogenase (LDH, EC: 1.1.1.27) released using a commercially available kit (LDH UV) (Labtest, Brazil).

The HepG2 cells were cultured and treated with the BDE-99 concentrations that presented the greatest effects in the viability and proliferation cell assays for 24 and 48 h. After cell exposure to BDE-99, the cell culture media were collected and the LDH released evaluated from the decrease in absorbance during 4 min in a Model U-2910 Hitachi spectrophotometer (Japan).

The LDH activity was calculated using the formula:

$$\text{LDH Activity} = [(\text{Abs}_{\text{time0'}} - \text{Abs}_{\text{time4'}})/\text{total time}] \times 8095$$

The cells were washed with PBS, trypsinised, incubated with the same volume of 0.4% (w/v) trypan blue solution for 3 min, and the viable (with no membrane damage) and non-viable (with membrane damage) cells counted using a light microscope and recorded (Altman et al., 1993).

2.9. Nuclear fragmentation

Nuclear fragmentation was assessed using the fluorescent dye Hoechst 33342. Briefly, HepG2 cells were seeded on coverslips at a density of 1×10^4 cells and treated with BDE-99 at the concentrations that presented the highest results in the viability and proliferation cell assays for 24 and 48 h. Each sample was tested with at least three replicates. The cells on the coverslips were then fixed with methanol at −20 °C for 2 h and then staining with 5 μg/mL Hoechst 33342 for 30 min at 37 °C (Holly, 2002).

Nuclear fragments were observed using a Leica DM 5000B fluorescence microscope (Germany) and 300 cells quantified per slide.

2.10. Caspase-9 and caspase-3 activities assay

Caspase-9 and caspase-3 activities were assayed using the caspase-3 fluorimetric assay kit and caspases-9 fluorimetric assay kit according to the manufacturer's instructions (Sigma–Aldrich). After 24 h treatment, cells were suspended in 50μL of lyses buffer, kept on ice for 15 min, and then centrifuged (14,000g, 15mim at 4 °C). The supernatants were collected for the assays. Activation of caspase-9 is based on hydrolysis of the substrate n-Acetyl-Leu-Glu-His-Asp7-amido-4-trifluoromethylcoumarin (Ac-LEHD-AFC) by caspase-9, resulting in the release of fluorescent 7-amino-4-trifluromethyl coumarin (AFC) moiety, while hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) by caspase-3, resulted in the release of the fluorescent 7-amino-4-methylcoumarin (AMC) moiety.

Reaction were performed in buffer containing supernatant proteins (50 μg/sample for caspase-9 and 25 μg/Sample for caspase-3) and caspase substrates, at 37 °C for 2 h, followed by fluorimetric detection using the excitation and emission wavelengths of 400/505 nm and 360/460 nm for caspase-9 and caspase-3, respectively.

2.11. Statistical analysis

The experimental data were evaluated using the analysis of variance (ANOVA), followed by the Dunnet test for the comparison of the various treated groups with their controls, using the GraphPrism program, version 5.1 for Windows. The results were considered statistically significant at $p < 0.05$.

3. Results

The results showed that the congener BDE-99 inhibited cell proliferation after 24 and 48 h of incubation, showing significant effects at the higher concentrations tested (18.22 ± 6.42% and 41.77 ± 10.5% for 10 μM and 25 μM, respectively) after 24 h of exposure. A significant effect was observed for concentrations as low as 0.5 μM when the cells were exposed to the compound for 48 h (Fig. 1).

Moreover, it was also demonstrated that the congener BDE-99 was able to induce a decrease in cell viability during both incubation periods for almost all the concentrations that lead to an inhibition of HepG2 cell proliferation (Fig. 2). These results demonstrated that there is a correlation between the effects observed in the first two experiments.

Fig. 3 shows the effect of BDE-99 on the mitochondrial membrane potential (MMP). The MMP also changed after exposure to 10 and 25 μM of the compound for 24 h. This effect was intensified after 48 h of incubation, showing significant effects in concentrations as low as 0.5 μM.

Similar results to those of the MMP assay were observed in the ROS accumulation test. Fig. 4 shows a significant increase in ROS accumulation after 24 h of incubation with BDE-99 at the highest concentration tested (25 μM). However when the effect was evaluated for 48 h, the exposure to 5 μM of the compound was sufficient to significantly increase ROS accumulation in the HepG2 cells.

To better understand the mechanism by which BDE-99 induces cell death, we evaluated the exposure of phosphatidyl serine on the outer cell membrane by assessing the FITC-annexin-V positive

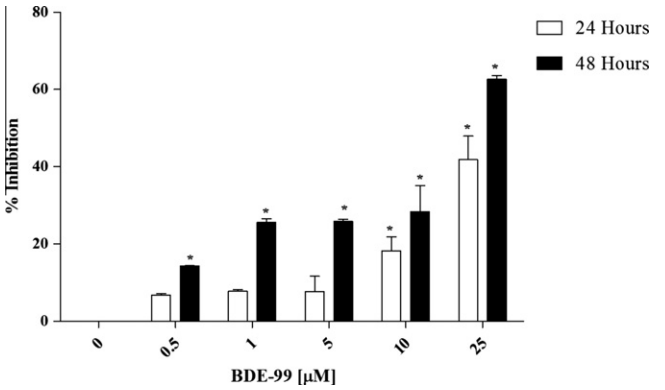


Fig. 1. The effects of BDE-99 (0.5–25 μM) on the proliferation of the HepG2 cell line after 24 and 48 h of exposure to the compound. Cell line proliferation was assessed using sulforhodamine B (SRB), as described in Section 2. The data are presented as the means ± SEM of a series of three experiments with different cell cultures. *Significantly different ($p < 0.05$) from the negative control (without BDE-99).

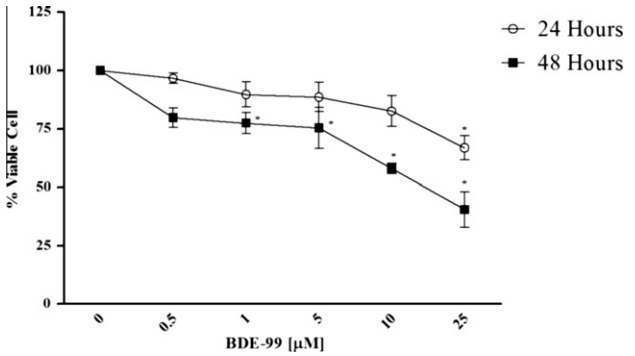


Fig. 2. The effects of BDE-99 (0.5–25 μM) on the viability of the HepG2 cell line after 24 and 48 h of exposure to the compound. Cell viability was assessed using (4,5 dimethylthiazol-2-il)-2,5 diphenyltetrazolium bromide dye (MTT assay), as described in Section 2. The data are presented as the means ± SEM of a series of three experiments with different cell cultures. *Significantly different ($p < 0.05$) from the negative control (without BDE-99).

cells. Fig. 5 demonstrates there was an increase in Annexin-V positive cells at the same concentrations that had decreased cell viability and proliferation after 24 h of exposure to BDE-99. It was also shown that after 48 h of exposure (Fig. 6) to this compound, concentrations starting at 5 μM were able to induce phosphatidyl serine exposure. On the other hand there was no increase in PI positive cells at any concentration or time tested.

In order to confirm these findings, the lactate dehydrogenase activity was assessed after 24 and 48 h of cell exposure to BDE-99. No difference was observed for any of the concentrations tested for either of the exposure times (data not shown), showing that the exposure to BDE-99 did not damage the cell membrane, which would allow the release of the cell contents. This effect was confirmed by the trypan blue exclusion assessment, which did not detect any significant damage to the cell membrane (data not shown).

Additionally, since exposure of phosphatidyl serine on the outer cell membrane is a caspase-dependent mechanism, we evaluated the caspases-9 and -3 activation after exposure to BDE-99.

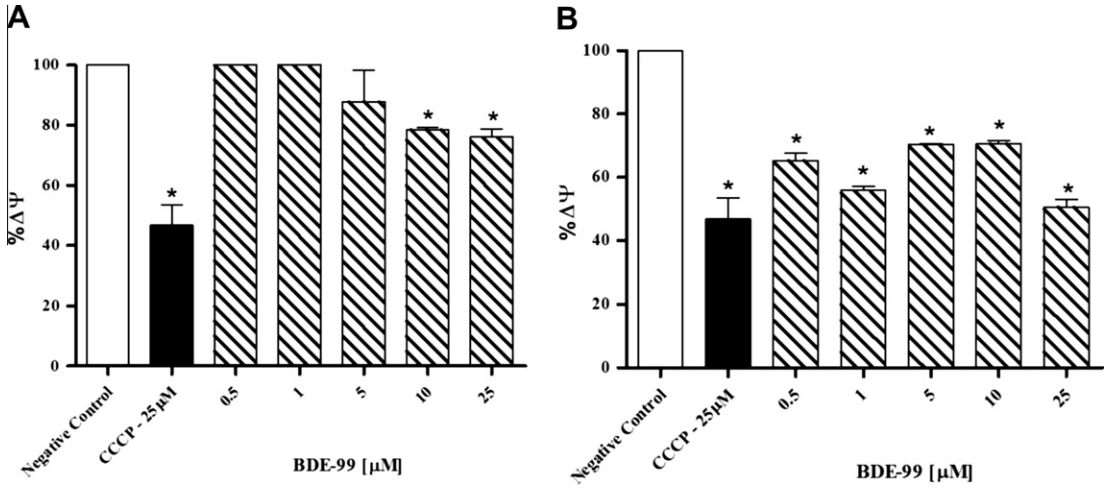


Fig. 3. The effects of BDE-99 (0.5–25 μM) on the mitochondrial membrane potential of the HepG2 cell line after 24 (A) and 48 h (B) of exposure to the compound. The mitochondrial membrane potential was assessed using TMRM (tetramethyrodamine, methyl ester) as described in Section 2. The data are presented as the means ± SEM of a series of three experiments with different cell cultures. *Significantly different ($p < 0.05$) from the negative control (without BDE-99). CCCP (25 μM) was used as the positive control.

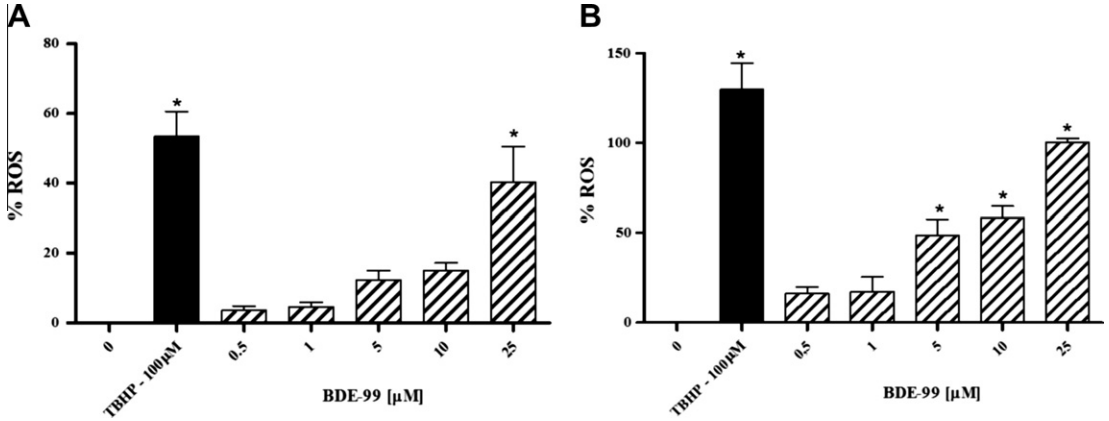
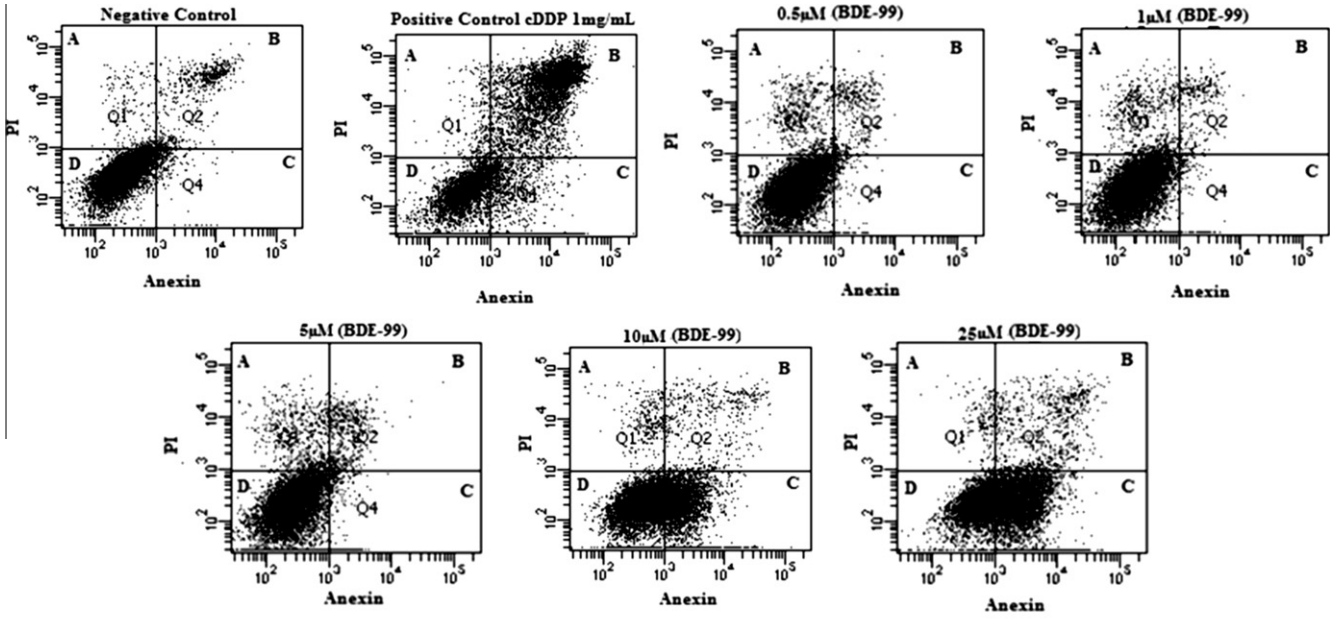


Fig. 4. The effects of BDE-99 (0.5–25 μM) on the induction of ROS accumulation in the HepG2 cell line after 24 (A) and 48 h (B) of cell exposure to the compound. ROS accumulation was assessed using the fluorescence probe 5,6-Chloromethyl-2′,7′-Dichlorodihydrofluorescein Diacetate, Acetyl Ester (CM-H_2DCFDA) as described in Section 2. The data are presented as the means ± SEM of a series of three experiments with different cell cultures. *Significantly different ($p < 0.05$) from the negative control (without BDE-99). Terc-butyl hydroperoxide (TBHP) 100 μM was used as the positive control.



	A	B	C	D
Negative Control	2,85±0.51	3.65±0.63	1.86±0,46	86.36±2,4
Positive Control (1mg/mL)	2,65±0.8	39.05±3.4*	15.27±2,59*	40.40±4.6*
0.5μM	5.25±0.9	3.65±0.63	3.25±0.71	88.3±2.04
1 μM	5.45±1.72	4.41±0.62	4.83±0.90	86.55±3.51
5μM	4.88±1.49	5.51±0.99	5.22±1.04	85.18±2.68
10μM	2.65±0.96	4.96±0.61	29.04±3.90*	61.18±6.07*
25μM	1.7±0.60	6.8±0.72	50.74±5.28*	38.97±3,72*

Fig. 5. The effects of BDE-99 (0.5–25 μM) on the exposure of phosphatidylserine by the HepG2 cell line after 24 h of cell exposure to the compound. Phosphatidylserine exposure was assessed by the Annexin-V (Anex.)/Propidium Iodide (PI) assay, as described in Section 2. The images are representative of three experiments with different cell cultures, A (PI^+ and $Anex^-$), B (PI^+ and $Anex^+$), C (PI^- and $Anex^+$) and D (PI^- and $Anex^-$) are presented as the means ± SEM of a series of three experiments. *Significantly different ($p < 0.05$) from the negative control (without BDE-99). A 1 mg/mL solution of cisplatin (cDDP) was used as the positive control.

Fig. 7A shows a significant increase in caspase-9 activity after incubation with 5, 10 and 25 μM of the compound for 24 h in a concentration-dependent manner, while Fig. 7B demonstrated that only exposure to 25 μM of BDE-99 induced a significant increase in caspase-3 activity in the same incubation period.

Finally, to confirm the induction of apoptosis suggested by the increase in annexin-V positive cells, we evaluated the nuclear fragmentation induced by BDE-99 by fluorescence microscopy, using the Hoechst 33342 dye. Fig. 8 demonstrates the presence of nuclear fragmentation after exposure to BDE-99 at concentrations of 10 and 25 μM for 24 h, with an increase in the amount of nuclear fragmentation with longer periods of incubation.

4. Discussion

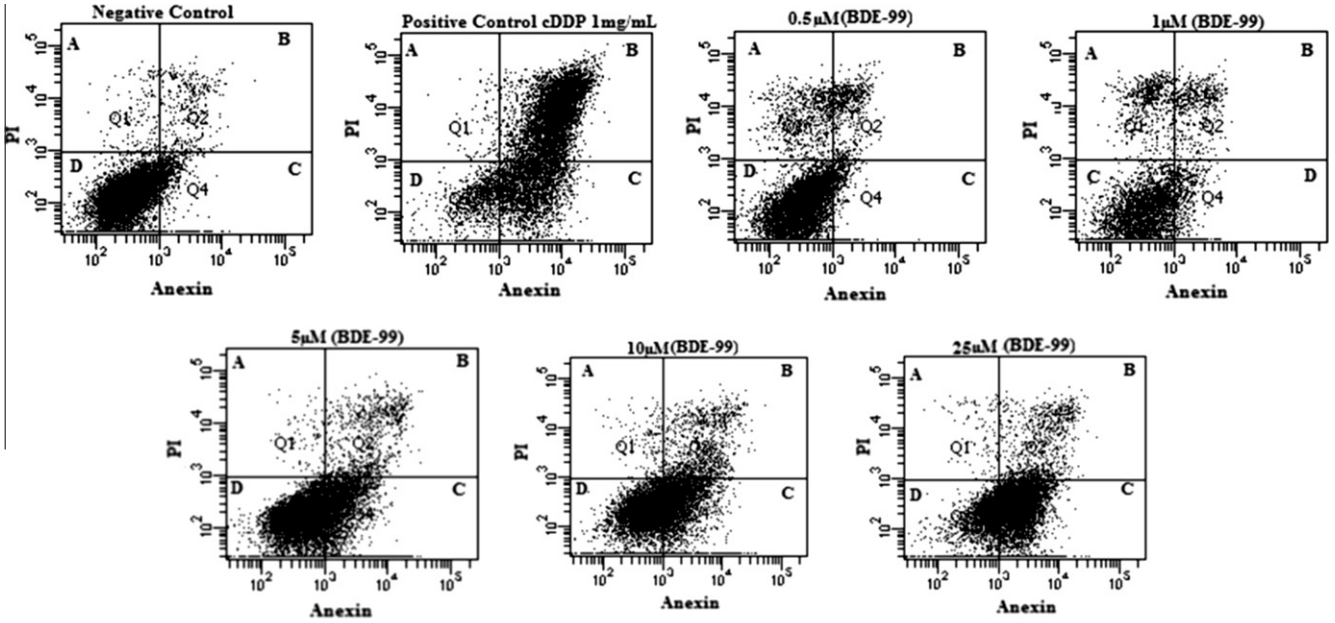
BDE-99 is a PBDE congener with little information about its toxicity to human health, and the mechanisms by which it can interfere with cell viability are still poorly understood. Since BDE-99 is one of the most common congeners found in the environment, it is an optimal candidate for toxicological evaluations, and in addition, PBDEs are resistant to degradation and can cause damage that will affect current and future generations.

Thus an evaluation of the interference with cell proliferation is a tool widely used to investigate the toxic mechanisms of different compounds, since it is an essential process for maintaining the homeostasis of living organisms. The effect on cell proliferation can occur by the inhibition of cell growth, leading to cell death, or by DNA damage with the subsequent production of a mutated cell with inappropriate proliferation and abnormal growth (Guo and Hay, 1999).

BDE-99 decreases HepG2 cell proliferation in a concentration-dependent manner that increases with the time of cell exposure to the compound. Moreover, BDE-99 decreased cell viability when assessed by the MTT assay at the same concentrations that interfered with cell proliferation, demonstrating that at least part of the observed effect on the proliferation was actually due to its ability to induce cell death.

It has already been described in the literature that other congeners, such as BDE-209 and BDE-47, decreased the number of cells with functional mitochondria as assessed by the same MTT method (Hu et al., 2007, 2009). These same groups also described the ability of BDE-47 and BDE-209 to induce apoptosis in HepG2 cells. BDE-99 has also been reported to induce cell death in cortical cultured cells at concentrations of 10 and 30 μM (Alm et al., 2010), the same range of concentration that we observed a decrease in HepG2 cell viability.

In order to better understand the mechanisms underlying BDE-99 toxicity and to observe if this congener would have the same ability to induce apoptosis in HepG2 cells, its ability to interfere with cell mitochondria was first measured. A decrease in the mitochondrial membrane potential was observed at almost all the concentrations that induced cell death (10–25 μM after 24 h of incubation and 0.5–25 μM after 48 h). This decrease in the mitochondrial membrane potential could occur due to an opening of the mitochondrial permeability transition pores, which would release proteins such as cytochrome *c*, that trigger the apoptotic pathway (Grivicich et al., 2007).



	A	B	C	D
Negative Control	1.3±0.36	5±1.23	9.27±2.67	84.45±3.1
Positive Control (1mg/mL)	1.3±0.83	72.9±10.4*	12.26±4.49	8.32±1.82
0.5μM	5.3±2.9	5.23±1.89	4.41±1.83	85.06±2.17
1 μM	6.55±1.48	5.78±1.67	6.31±1.65	83.26±4.28
5μM	2.17±0.82	6.86±0.67	31.03±7.06*	60.31±6.65*
10μM	1.4±0.31	9.18±1.96	41.35±5.60*	48.2±6.24*
25μM	0.62±0.17	7.02±0.59	67.28±6.96*	24.27±6.04*

Fig. 6. The effects of BDE-99 (0.5–25 μM) on the exposure of phosphatidylserine by the HepG2 cell line after 48 h of cell exposure to the compound. Phosphatidylserine exposure was assessed by the Annexin-V (Anex.)/Propidium Iodide (PI) assay, as described in Section 2. The images are representative of three experiments with different cell cultures, A (PI^+ and $Anex^-$), B (PI^+ and $Anex^+$), C (PI^- and $Anex^+$) and D (PI^- and $Anex^-$) are presented as the means ± SEM of a series of three experiments. *Significantly different ($p < 0.05$) from the negative control (without BDE-99). A 1 mg/mL solution of cisplatin (cDDP) was used as the positive control.

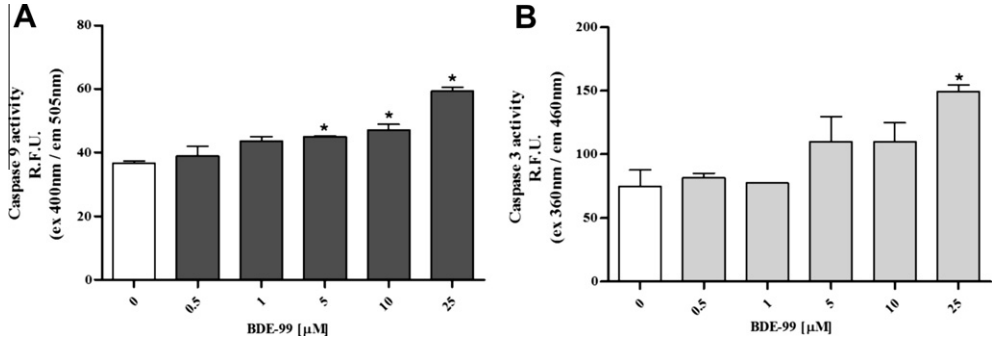


Fig. 7. The effects of BDE-99 (0.5–25 μM for 24 h) on caspase-9 and caspase-3 activities. Caspases activities were assessed using the commercially available fluorimetric assay kits as described in Section 2. Relative Fluorescence Unit (R.F.U.) was evaluated for each assay. The data are presented as the means ± SEM of a series of three experiments with different cell cultures. _. *Significantly different ($p < 0.05$) from the negative control (without BDE-99).

Our results also showed a clear relationship between a decrease in the mitochondrial membrane potential and accumulation of ROS. Therefore, just as observed for the BDE-209 and BDE-47 congeners, the toxic effects of BDE-99 are related to ROS accumulation (Hu et al., 2007; Huang et al., 2010; Shao et al., 2008; Weihong et al., 2008).

We also evaluated the ability of BDE-99 to induce apoptosis. Apoptotic cell death is associated with characteristics such as phosphatidylserine exposure due to selective oxidation (Tyurina et al., 2000; Matsura et al., 2005). Our results showed that high levels of ROS induced by cell exposure to BDE-99 were followed by phosphatidylserine exposure, suggesting that ROS accumulation induced by BDE-99 can lead to apoptosis.

In addition, the LDH leakage studies showed no increase in LDH after exposure to BDE-99, which together with the absence of PI stained cells and the continued ability of the cells to exclude trypan blue, suggests that the necrosis pathway is not relevant in BDE-99 induced HepG2 toxicity. However, there is controversy about the

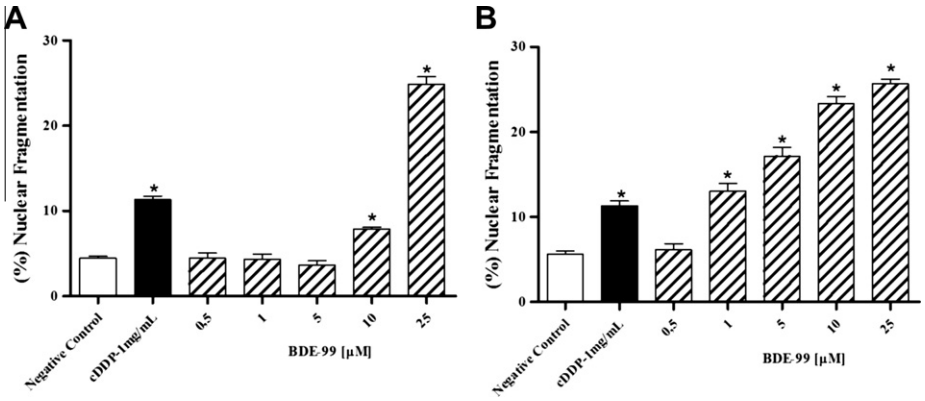


Fig. 8. The effects of BDE-99 (0.5–25 μM for 24 h) on nuclear fragmentation of the HepG2 cell line. Nuclear Fragmentation was assessed using the fluorescent dye Hoechst 33342 using fluorescence microscopy as described in Section 2. The data are presented as the means ± SEM of a series of three experiments with different cell cultures. *Significantly different ($p < 0.05$) from the negative control (without BDE-99). A 1 mg/mL solution of cisplatin (cDDP) was used as the positive control.

ability of PBDEs to induce LDH leakage. BDE-47 and BDE-209 were reported to cause a concentration-dependent inhibition of MTT reduction and LDH leakage in human neuroblastoma cells (He et al., 2008), and HepG2 cells (Hu et al., 2007), whereas BDE-99 (up to 100 μM) did not induce the release of LDH in human astrocytoma cells cultured for 24 h, even though the MTT had decreased significantly (Madia et al., 2004). This last finding is in agreement with the present results for the same BDE congener.

The present results also confirmed that the apoptotic pathway was induced by HepG2 exposure to BDE-99 by assessing other characteristics of this process, that is chromatin condensation and DNA fragmentation, which were assessed by fluorescence microscopy using the dye Hoescht 33342, and an increased amount of DNA fragmentation was observed in a concentration-dependent manner. Furthermore, apoptotic pathway was also confirmed by the evaluation of caspases activities, which showed an increased activation of caspase-9 and caspase-3 after 24 h of exposure to the compound.

Other published works had already demonstrated that some PBDEs congeners could induce apoptosis *in vitro*, such as BDE-209 in HepG2 cells (Hu et al., 2007), however this is the first time that it was shown that the induction of apoptosis by BDE-99 in HepG2 cells might involve mitochondrial dysfunction.

In summary, the present research contributed data that helps clarify the toxicity of PBDEs. These results showed that the congener BDE-99 induced cell death in HepG2 cells by the apoptosis pathway, interfering with the mitochondrial membrane potential and inducing the accumulation of ROS.

Despite the above proposed toxic mechanism of BDE-99, it should be considered that PBDEs can be metabolized in the liver, producing less brominated metabolites and oxidative metabolites, such as hydroxylated BDE congeners, which can present higher toxicity than the original compound (Dong et al., 2010). So, studies with the PBDEs metabolites need to be done in order to better understand the extension of their toxicity to humans.

Conflict of interest statement

The authors have no conflicts of interest to declare.

Acknowledgments

FAPESP (Proc. N° 2009/06912-6) and CAPES, Brazil, supported this work. We thank Professor Carlos Curti PhD. and the Biochemistry Laboratory at the Faculty of Pharmaceutical Sciences of Ribeirão Preto/Brazil for the technical support.

References

Albina, M.L., Alonso, V., Linares, V., Bellés, M., Sirvent, J.J., Domingo, J.S., Sánchez, D., 2010. Effects of exposure to BDE-99 on oxidative status of liver and kidney in adult rats. Toxicology 271, 51–56.

Alm, H., Scholz, B., Kultima, K., Nilson, A., Andrén, P.E., Savitski, M.M., Bergman, A., Stigson, M., Fex-Svenningsen, A., Dencker, L., 2010. In vitro neurotoxicity of PBDE-99: immediate and concentration-dependent effects on protein expression in cerebral cortex cells. Journal of Proteome Research 9, 1226–1235.

Altman, S.A., Randers, L., Rao, G., 1993. Comparison of trypan blue dye exclusion and fluorometric assays for mammalian cell viability determinations. Biotechnology Progress 9, 671–674.

Branchi, I., Capone, F., Alleva, E., Costa, L.G., 2003. Polybrominated diphenyl ethers: neurobehavioral effects following developmental exposure. Neurotoxicology 24, 449–462.

Chernyak, B.V., Izymov, D.S., Lyamzaev, K.G., Pashkovskaya, A.A., Pletjushkina, O.Y., Antonenko, Y.N., Sakharov, D.V., Wirtz, K.W.A., Skulachev, V.P., 2006. Production of reactive oxygen species in mitochondria of HeLa cells under oxidative stress. Biochimica et Biophysica Acta 1757, 525–534.

Costa, L.G., Giordano, G., 2007. Developmental neurotoxicity of polibrominated diphenyl ethers (PBDE) flame retardant. Neurotoxicology 28, 1047–1067.

Costa, L.G., Giordano, G., Tagliaferri, S., Caglieri, A., Mutti, A., 2008. Polybrominated diphenyl ether (PBDE) flame retardant: environmental contamination, human body burden and potential adverse health effects. Acta Biomedica 79, 172–183.

Covaci, A., Roosens, L., Dirtu, A.C., Waegeneers, N., Overmeire, I.V., Neels, H., Goeyens, L., 2009. Brominated flame retardant in Belgian home-produced eggs: level and contaminant sources. Science of the Total Environment 407 (11), 4387–4396.

Denizot, F., Lang, R., 1986. Rapid colorimetric assay for cell growth and survival modifications to the tetrazólium dye procedure giving improved sensitivity and reability. Immunological Methods 89, 271–277.

Dong, H., Li, Z., Man, X., Zhou, J., Lu, H., Wang, S., 2010. Identification of the metabolites of polybrominated diphenyl ether 99 and its related cytochrome P450s. Journal of Biomedical Research 24 (3), 223–232.

Gandhi, N., Bhavsar, S.P., Gewurtz, S.B., Tomy, G.T., 2011. Can biotransformation of PBDE 99 in lake trout cause bioaccumulation of more toxic lower brominated PBDE (PBDE 47, 99) over a long term? Environment International 37, 170–177.

Grivicich, I., Regner, A., Rocha, A.B., 2007. Morte Celular por Apoptose Revista Brasileira de Cancerologia 53 (3), 335–343.

Guo, M., Hay, B., 1999. Cell proliferation and apoptosis. Current Opinion in Cell Biology 11, 745–752.

Hardy, M.L., 1999. Regulatory status and environmental properties of brominated flame retardants undergoing risk assessment in the EU: DBDPO, OBDPO, PeBDPO and HBCD. Presented at 6th European Meeting on Fire Retardancy of Polimeric Materials. Polymer Degradation and Stability 64, 545–556.

He, W., He, P., Wang, A., Xia, T., Xu, B., Chen, X., 2008. Effects of BDE-47 on cytotoxicity and genotoxicity in human neuroblastoma cells in vitro. Mutation Research 649, 62–70.

Hites, R.A., 2004. Polybrominated diphenyl ethers in the environment and in people: a meta-analysis of concentrations. Environmental Science and Technology 38, 19–28.

Holly, J.M., 2002. Curcumin disrupts mitotic spindle structure and induces micronucleation in MCF-7 breast cancer cells. Mutation Research 519, 71–84.

Huang, H.C., Giordano, G., Costa, L.G., 2010. Comparative Cytotoxicity and intracellular accumulation of five polybrominated diphenyl congeners in mouse cerebellar granule neurons. Toxicological Sciences 114 (1), 124–132.

Hu, X.Z., Xu, Y., Hu, D.C., Hui, Y., Yang, F.X., 2007. Apoptosis induction on human hepatoma cells Hep G2 of decabrominated diphenyl ether (PBDE-209). Toxicology Letters 171, 19–28.

Hu, X., Hu, D., Xu, D., 2009. Effects of tetrabrominated diphenyl ether and hexabromocyclododecanes in single and complex exposure to hepatoma HepG2 cells. Environmental Toxicology and Pharmacology 27, 327–337.

Imberti, R., Nieminen, A.-L., Herman, B., Lemasters, J.J., 1993. Mitochondrial and glycolytic dysfunction in lethal injury to hepatocytes by *t*-butylhydroperoxide: protection by fructose, cylclosporin A and trifluoperazine. Journal of Pharmacology and Experimental Therapeutics 265, 392–400.

Jing, A., Shuhui, L.Z., Yipei, W., Kewen, Z., Xinyu, Z., Yangjun, W., Minghong, W., Zhiqiang, Y., Guoying, S., Jiamo, F., Yuecheng, H., 2010. The cytotoxic effects of synthetic 6-hydroxylated and 6-methoxylated polybrominated diphenyl ether 47 (PBDE47). Environmental Toxicology 26 (6), 591–599.

Knasmuller, S., Parzefal, W., Sanyal, R., Ecker, S., Schwab, C., Uhl, M., Mersch-Sundermann, V., Willianison, G., Hietsch, G., Langer, T., Darroudi, F., Natarajan, A.T., 1998. Use of metabolically competent human hepatom cell for the detection of mutagens and antimutagens. Mutation Research 402, 185–202.

Letcher, R.J., Bustnes, J.O., Dietz, R., Jenssen, B.M., Jorgensen, E.H., Sonne, C., Verreault, J., Vijayan, M.M., Gabrielsen, G.W., 2010. Exposure and effects assessment of persistent organohalogen contaminants in arctic wildlife and fish. Science of the Total Environment 408, 2995–3043.

Li, C., Yu, H., Zhao, Y., Zhang, G., Wu, Y., 2008. Level of polybrominated diphenyl ethers (PBDEs) in breast milk from Beijing. Chemosphere 78, 182–186.

Ma, S., Yu, Z., Zhang, X., Ren, G., Peng, P., Sheng, G., Fu, J., 2012. Level and congeners profiles of polybrominated diphenyl ethers (PBDEs) in breast milk from Shanghai: implication for exposure route of higher brominated BDEs. Environment International 42, 72–77.

Madia, F., Giordano, G., Fattori, V., Vitalone, A., Branchi, I., Capone, F., Costa, L.G., 2004. Differential in vitro neurotoxicity of the flame retardant PBDE-99 and of the PCB Aroclor 1254 in human astrocytoma cells. Toxicology Letters 154, 11–21.

Matsura, T., Togawa, A., Kai, M., Nishida, T., Nakada, J., Ishibe, Y., Kojo, S., Yamamoto, Y., Yamada, K., 2005. The presence of oxidized phosphatidylserine on Fas-mediated apoptotic cell surface. Biochimica at Biophysica Acta 1736, 181–185.

McDonald, T.A., 2002. A perspective on the potential health risk of PBDE. Chemosphere 46, 745–755.

Shao, J., White, C.C., Dabrowski, M.J., Kavanagh, T.J., Eckert, M.L., Gallagher, E.P., 2008. The role of mitochondrial and oxidative injury in BDE 47 toxicity to human fetal liver hematopoietic stem cell. Toxicological Sciences 101 (1), 81–90.

Skehan, P., Storeng, R., Scudiero, D., Monks, A., Mcmahon, J., Vistica, D., Warren, J.T., Bokesch, H., Kenney, S., Boyd, R.M., 1990. New colorimetric cytotoxicity assay for anticancer-drug screening. Journal of the National Cancer Institute 82, 1107–1112.

Shen, H., Ding, G., Han, G., Wang, X., Xu, X., Han, J., Lou, X., Xu, C., Cai, D., Song, Y., Lu, W., 2010. Distribution of PCDD\Fs, PCBs, PBDEs and organochorine residues in children's blood from Zheijiang. Chemosphere 80, 170–175.

Talsness, C.E., 2008. Overview of toxicological aspects of polybrominated diphenyl ethers: a flame retardant additive in several consumer products. Environmental Research 108, 158–167.

Toms, A.R., Foran, F.A., Symons, R.K., Burniston, D., Furst, P., Muller, J.F., 2007. Polybrominated diphenil ethers (PBDEs) in human milk from Australia. Chemosphere 68, 797–803.

Tyurina, Y.Y., Shvedova, A.A., Kawai, K., Tyurin, V.A., Komminemi, C., Quinn, P.J., Fabisiak, J.P., Kagan, V.E., 2000. Phospholipid signaling in apoptosis: peroxidation and externalization of phosphatidylserine. Toxicology 148, 93–101.

Verner, M.-A., Bouchard, M., Fritsche, E., Charbonneau, M., Haddad, S., 2011. In vitro neurotoxicity data in human risk assessment of polybrominated diphenyl ethers (PBDEs): overview and perspectives. Toxicology in Vitro 25 (8), 1509–1515.

Weihong, H., Ping, H., Aiguo, W., Tao, X., Bayi, X., Xuemim, C., 2008. Effects of PBDE-47 on citotoxicity and genotoxicity in human neuroblastoma in vitro. Genetic Toxicology and Environmental Mutagenesis 649, 62–70.

Yan, C., Huang, D., Zhang, Y., 2011. The involvement of ROS over production and mitochondrial dysfunction in PBDE 47 induced apoptosis on Jurkat cells. Experimental and Toxicology Pathology 63, 413–417.

Zhang, Y., Guo, G.L., Han, X., Zhu, C., Kilfoy, B.A., Zhu, Y., Boyle, P., Zheng, T., 2008. Do polybrominated diphenyl ethers (PBDE) increase the risk of thyroid cancer? Bioscience Hypotheses 1, 195–199.

Zhivotovsky, B., Samali, A., Galm, A., Orrenius, S., 1999. Caspases: their intracellular localization and translocation during apoptosis. Cell Death and Differentiation 6, 644–651.